Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/CA05/000108

International filing date: 27 January 2005 (27.01.2005)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/539,556
Filing date: 27 January 2004 (27.01.2004)

Date of receipt at the International Bureau: 22 April 2005 (22.04.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

PCT/CA 20050000108

PA 1290977

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

March 09, 2005

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: ***60/539,556***
FILING DATE: ***January 27, 2004***

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

E. BORNETT
Certifying Officer

Substitute Form PTO/SB/16 (11-95)
Approved for use through 01/31/98, OMB 0651-0037
Patent and Trademark Office; US DEPARTMENT OF COMMERCE

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 C.F.R. § 1.53(c).

Docket Number	50037/003002	Type a plus sign (+) inside this box --->	+

INVENTOR(S)/APPLICANT(S)

LAST NAME	FIRST NAME	MIDDLE INITIAL	RESIDENCE (CITY AND EITHER STATE OR FOREIGN COUNTRY)
Miller	Freda		Toronto, Canada
Akhavan	Mahnaz		Toronto, Canada
Craig	Laura		Toronto, Canada
Fernandes	Karl		Toronto, Canada
McKenzie	Ian		Toronto, Canada
Toma	Jean		Toronto, Canada

TITLE OF THE INVENTION (280 characters max)

SKIN-DERIVED STEM CELLS AND USES THEREOF

CORRESPONDENCE ADDRESS

Kristina Bieker-Brady, Ph.D.
Clark & Elbing LLP
101 Federal Street
Boston, MA 02110
Customer No.: 21559

STATE	MA	ZIP CODE	02110	COUNTRY	USA

ENCLOSED APPLICATION PARTS (check all that apply)

☒ Specification	Number of pages: 24	☒ Cover Sheet: 1 page	
☒ Claims	Number of pages: 5	☐ Other (specify):	
☒ Drawings	Number of sheets: 7		

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☒ A check or money order is enclosed to cover the filing fees. ☒ The Commissioner is hereby authorized to charge filing fees not covered and apply any credits to Deposit Account Number: 03-2095	FILING FEE AMOUNT	$80.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are:

☒ Applicant claims small entity status under 37 C.F.R. § 1.27.

Respectfully submitted,

SIGNATURE: K. Bieker-Brady DATE: January 27, 2004

TYPED OR PRINTED NAME: Kristina Bieker-Brady, Ph.D.

REGISTRATION NO.: 39,109

Certificate of Mailing

Date of Deposit: January 27, 2004 Label Number: EV320807390US

I hereby certify under 37 C.F.R. § 1.10 that this correspondence is being deposited with the United States Postal Service as **"Express Mail Post Office to Addressee"** with sufficient postage on the date indicated above and is addressed to Mail Stop Provisional Patent Application, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Elvis De La Cruz
Printed name of person mailing correspondence

Signature of person mailing correspondence

PROVISIONAL APPLICATION

UNDER 37 C.F.R. § 1.53(c)

APPLICANTS : FREDA MILLER, MAHNAZ AKHAVAN, LAURA CRAIG, KARL FERNANDES, IAN MCKENZIE, AND JEAN TOMA

TITLE : SKIN-DERIVED STEM CELLS AND USES THEREOF

SKIN-DERIVED STEM CELLS AND USES THEREOF

Field of the Invention

In general, the present invention relates to the field of stem cell biology.

Background of the Invention

While adult mammalian stem cells were previously thought only to differentiate into cells of their tissue of origin, a number of recent reports have identified cultured adult stem cells that show a surprisingly diverse differentiation repertoire (1). Although at least some reports of multipotency are due to unanticipated cellular fusion events that occurred in vivo (2-4), compelling evidence still exists for the multipotency of a number of cultured adult stem cell populations. Perhaps the most striking examples of this multipotency derive from blastocyst injection studies, where both MAPC cells isolated after long-term culture of bone marrow cells5 and neural stem cells from the CNS (6) contributed to many different developing tissues. However, one caveat to these studies is that this multipotency was demonstrated only after these stem cell populations had been expanded for significant periods of time in culture, raising the question of whether these results truly reflect previously unsuspected endogenous multipotent adult precursors, or whether they are the consequence of culture-induced dedifferentiation and/or reprogramming (1).

In this regard, we have previously identified one such multipotent precursor cell population from adult mammalian dermis (7). These cells, termed SKPs for Skin-derived Precursors, can be isolated and expanded from rodent and human skin, and differentiate into both neural and mesodermal progeny, including into cell types that are never found in skin, such as neurons. Interestingly, one endogenous embryonic stem cell population that has a similar broad differentiation potential and that contributes to the dermis are neural crest stem cells (NCSCs) (8). Based upon this similarity in potential, we tested the

hypothesis that SKPs represent a multipotent neural crest-like precursor cell that colonizes mammalian tissues during embryogenesis, and that persists into adulthood. Here, we provide evidence supporting this hypothesis, and further identify a dermal niche for these endogenous precursors.

Summary of the Invention

We have previously described the isolation of multipotent stem cells from juvenile and adult rodent skin, which we have termed as SKPs for *sk*in-derived *p*recursors. These cells derive from the dermis, and clones of individual cells proliferate and differentiate in culture to produce neurons, glia, smooth muscle cells and adipocytes. These cells have previously been described in Patent Application Nos. WO/0153461 and WO/03010243, hereby incorporated by reference.

Here, we show that multipotent stem cells are also found in the dermal papilla of hair follicles. Based on our discovery, the present invention features methods for purifying multipotent stem cells from the dermal papilla of hair follicles. Briefly, hair follicles (or portions of hair follicles containing the follicular dermal papilla) are obtained from a mammal. If desired, hair follicles may be further dissociated into smaller pieces by any method including, for example, enzymatic digestion or mechanical disruption. For example, the hair follicle may be dissociated to obtain the follicular dermal papilla. Hair follicles or portions thereof are next cultured in conditions under which multipotent stem cells grow and proliferate non-adherently and non-multipotent stem cells die or adhere to the culture substrate. Under such conditions, multipotent stem cells typically grow as part of floating three-dimensional structures. To collect multipotent stem cells, the non-adherent cells are separated from adherent cells. If desired, these non-adherent cells which contain the multipotent stem cells are further cultured in the same conditions as described above and collected following their separation from adherent cells until at least 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 99%, or even 100% of cells are multipotent stem cells (or progeny thereof).

Desirably, the multipotent stem cells of the invention express at least one, two, three, or more than three of the following molecular markers: nestin, WNT-1, vimentin, versican, fibronectin, S100, slug, snail, twist, Pax3, Sox9, Dermo, and SHOX2. Such markers may be detected by any standard method known in the art including, for example, Northern blot analysis, RT-PCR, Western Blot analysis, *in situ* hybridization, and immunohistochemical analysis. Such methods are described in detail, for example, in F. Ausubel *et al.*, *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, NY, 1998, hereby incorporated by reference. The multipotent stem cells of the invention may also express increased levels (at least one-fold, two-fold, three-fold, or more) of slug, snail, twist, and Pax3 relative to central nervous system neural stem cells. Desirably, the multipotent stem cells of the invention do not express measurable levels of at least one, two, three, or more than three of the following molecular markers: tyrosinase, c-kit, tryp-1, and DCT, which are markers of melanoblasts and melanocytes. The multipotent stem cells may also not express measurable levels of one or more of the following markers of Schawnn cells: MBP, PO, p75NTR, or SOX10. According to this invention, the level of a marker in a cell is considered as being "not measurable" if the marker in the cell cannot be detected by a method that under appropriate conditions detects the expression of the corresponding marker in a control cell. For example, the multipotent stem cells of the invention is considered not to express measurable levels of P75NTR since using the same RT-PCR analysis, p75NTR expression is undetectable in the cells of the invention but detectable in CNS neural stem cells.

Under appropriate conditions, multipotent stem cells purified from the dermal papilla of hair follicles may differentiate into various non-neural (e.g., hair follicle cell, bone cell, smooth muscle cell, or adipocyte) and neural cell (a neuron, an astrocyte, a Schwann cell, or an oligodendrocyte) types. Accordingly, the present invention provides methods for inducing hair growth by providing to a mammal a population of cells, in which at least 30% of cells are stem cells purified from the dermal papilla of hair follicles. Alternatively, multipotent stem cells may be purified from the dermal papilla of hair follicles and following their differentiation into hair follicle cells *in vitro*, these cells

are provided to the mammal being treated. According to this invention, cells may be provided to a mammal using any standard method in the art, such as those described, for example, in Unger *et al, Skin Therapy Lett.* (2003) 8: 5-7. Accordingly, hair growth may be induced anywhere on the skin of the mammal (such as the head, face, legs, or arms of a mammal), even in areas where hair follicles are not usually found. Desirably, hair growth is induced in areas in which hair was previously present but has been lost. Mammals being treated according to the present invention may have a condition characterized by loss or lack of hair, including for example, alopecia, male pattern baldness, female pattern baldness, accidental injury, damage to hair follicles, surgical trauma, burn wound, radiation or chemotherapy treatment site, incisional wound, and donor site wound from skin transplant and ulcer. Alternatively, the mammal being treated may simply have a desire to modify physical appearance. Desirably, hair is induced by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 100% relative to an untreated control.

In all foregoing aspects of the invention, multipotent hair stem cells may be purified from the dermal papilla of hair follicles from any post-natal mammal, including an adult or juvenile mammal (e.g., human). If these cells are used in transplantation procedures, the donor mammal is desirably immunologically similar to the recipient mammal. Even more desirably, these cells are obtained from an autologous source. Optionally, the cell of the invention may contain a heterologous gene in an expressible genetic contruct. Such a gene may encode, for example, a therapeutic protein or a protein which induces or facilitates differentiation of the stem cell.

Brief Description of the Figures

Figure 1. SKPs are a distinct stem cell population that expresses markers of embryonic neural crest. a, Immunocytochemical analysis of SKPs spheres cultured from neonatal mouse (nestin, fibronectin) or rat (p75NTR) skin, and passaged two or three times (top panels) as compared to neurospheres cultured from the embryonic

telencephalon and passaged a similar number of times (bottom panels). In all panels, the immunostained cells are red, and the blue is from Hoechst staining of the nuclei to show all cells. SKPs express fibronectin but not p75NTR, whereas neurospheres do not express fibronectin, but do express p75NTR. **b**, RT-PCR for genes involved in embryonic neural crest determination and migration in total RNA isolated from SKPs spheres compared to embryonic telencephalic neurospheres (CNS), both of which were cultured in the presence of FGF2 plus EGF. The positive control (+ve) was RNA from E12 neural tube. RT-PCR for GAPDH was used as a loading control, and the last lane (H2O) was run as a negative control. **c**, RT-PCR for Dermo-1 and SHOX2, transcription factors involved in dermal and craniofacial development, in RNA from SKPs and CNS neurospheres as described in **b**. The positive control (+ve) was RNA from E16 forelimb. **d**, Immunocytochemical analysis for markers of melanoblasts and hematopoietic stem cells. The two left panels were immunostained with antibodies for nestin (red, arrows) and for the melanoblast marker tryp1 (green, arrowheads), with the top panel being total dissociated skin cells, and the bottom a primary SKPs sphere. Note that the tryp1-positive and nestin-positive skin cell populations are distinct, and that primary SKPs spheres express nestin but not tryp1. The two right panels were immunostained for c-kit (green), a marker for both melanoblasts and hematopoietic stem cells, with the top panel being a cytospin of a bone marrow aspirate (positive cells are marked with arrowheads), and the bottom panel a primary SKPs sphere, which is not immunoreactive. In all panels, the blue derives from the Hoechst staining of cell nuclei.

Figure 2. SKPs differentiate into peripheral neurons and Schwann cells. a, RT-PCR for two markers of peripheral catecholaminergic neurons, dopamine-β-hydroxylase mRNA (DβH) and peripherin mRNA in murine SKPs differentiated for 1 week in 10% serum (Diff.). SKPs spheres and dissociated SKPs plated in FGF2 and EGF (Prolif.) do not express these mRNAs. **b**, Western blot analysis for NCAM and dopamine-β-

hydroxylase in murine SKPs spheres versus SKPs differentiated for 14 days in 10% serum supplemented with neurotrophins. The positive control was protein isolated from cultured peripheral sympathetic neurons from the superior cervical ganglion (SCGs). **c**, Immunocytochemical analysis of differentiated murine SKPs for markers of peripheral neurons. (Left top panel) Morphologically complex differentiated cells coexpress the neuronal markers βIII-tubulin and NFM (yellow cells in the merged image). (Right top panel) Differentiated cells coexpress βIII-tubulin (red, top inset) and p75NTR (green, bottom inset), proteins that are expressed by virtually all peripheral neurons. (Bottom left panel) A subset of differentiated cells also express tyrosine hydroxylase (TH). **d**, RTPCR for three markers of peripheral Schwann cells, p75NTR, myelin basic protein (MBP) and P0 peripheral myelin protein (P0) in total RNA from undifferentiated and differentiated rat SKPs. **e**, Immunocytochemical analysis of differentiated SKPs, showing that a subset of bipolar cells coexpress (left panel) S100beta (red) and MBP (green), or (right panel) S100beta (red) and GFAP (green). In panels **c** and **e**, the blue derives from Hoechst stained nuclei of all cells in the field.

Figure 3. Developing and adult skin contain neuronal precursors. a, b, Immunocytochemical analysis for neuron-specific âIII-tubulin in primary E18 (**a**, middle panel) or adult (**a**, right panel) murine skin cells versus E18 murine CNS telencephalic cells (**a**, left panel). Cells from all three sources were plated in FGF2 and 10% serum for 7 days and then switched to media containing 5% serum supplemented with neurotrophins. **b,** Double-labelling of differentiated primary E18 murine skin cells with antibodies for βIII-tubulin (green) and NFM (red) (cells are yellow in the merged image). More cells express βIII-tubulin than NFM, since these are early and late neuronal markers, respectively. **c,** Western blot analysis for proteins expressed in peripheral neurons in E18 skin cells differentiated (Diff. SCs) under conditions similar to those in panel **a**. For comparison, equal amounts of protein were analysed from lysates of skin at the same age, E18 skin cells that were not differentiated (SCs), and from primary cultures

of peripheral sympathetic neurons (SCG). In panels **a** and **b**, the blue derives from Hoechst stained nuclei.

Figure 4. Multipotent endogenous SKPs are abundant in skin during late embryogenesis and persist into adulthood. a, Phase contrast micrographs of primary murine SKPs spheres grown for 1 week at varying concentrations of starting cells (c/ml). At concentrations from 25,000 to 100,000 cells/ml the number of SKPs spheres generated is linear with concentration. **b,** Phase contrast micrograph of primary SKPs spheres grown immobilized in methylcellulose. **c,d,** Quantitation of the number of cells that give rise to primary SKPs spheres in murine back skin isolated at various developmental ages ranging from embryonic day 13 to adulthood. In panel **c** cell numbers are expressed relative to a given number of primary skin cells, whereas in panel **d** numbers are normalized to the total number of cells present in a back skin isolation. **e,** immunocytochemical analysis for nestin expression in a primary murine SKPs clone plated onto poly-d-lysine/laminin. Note that these primary spheres express nestin and fibronectin, and that the nestin-positive cells migrate out of the sphere when differentiated. **f,** Immunocytochemical analysis of three different primary murine SKPs clones, one for the neuronal marker βIII-tubulin (left panel), one for βIII-tubulin (green) and smooth muscle actin (red, SMA) (middle panel), and one for SMA (red) and the glial cell marker GFAP (green) (right panel), demonstrating that these primary SKPs clones can generate both neural and mesodermal progeny. In panels **e** and **f**, the blue is from Hoechst-stained nuclei.

Figure 5. Skin expression of embryonic SKPs transcription factors is localized to the dermal papillae of hair and whisker follicles. a, RT-PCR for the SKPs markers nestin, twist and slug in developing (E17 and P2) and adult skin. **b-f,** E18.5 dorsal follicle dermal papillae stained with (**b**) hematoxylin and eosin, (**c**) endogenous alkaline phosphatase (blue) and keratin-5, which stains basal epidermis and hair follicles), (**d**)

nexin, (**e**) slug, and (**f**) snail. **g-j**, Postnatal anagen follicles identified by morphology and marker gene expression have dermal papillae that express (**g**) snail at P2 (arrowhead), and (**h**) slug at P8, when all follicles are entering their first synchronized anagen phase. After the second wave of anagen, the hair follicle cycles by P36 are becoming asynchronous, but these late anagen dermal papillae still express (**i**) nexin and (**j**) twist (arrowheads). **k,l,** Despite differences in developmental programs, developing E16.5 vibrissae still express dermal papilla markers such as (**k**) nexin and neural crest markers such as snail (**l**).

Figure 6. Adult vibrissae and dermal papillae contain SKPs-like cells. a, Immunocytochemical analysis of dissociated adult rat vibrissal cells that were plated in media containing FGF2 and 10% serum for 1 (1d), 4 (4d) or 7 (7d) days. The top row are phase contrast micrographs and the bottom row the nestin immunostains of the same fields. Under these conditions, clusters of nestin-positive cells proliferate, and then start to differentiate morphologically. **b,c,** βIII-tubulin immunoreactivity in dissociated vibrissal cells (b) or vibrissal explants (c) after culturing for 7 days as in panel **a**. **d,** RT-PCR for the dermal papilla markers nexin, versican and Wnt-5a in total RNA isolated from E12 embryos (+ve), skin (S), SKPs spheres (SKP) or CNS neurospheresβ(CNS). Note that SKPs but not neurospheres express all of these markers. **e,** Bright field microscopy of an intact vibrissa (top) and the separated vibrissa and papilla (bottom). The arrowhead indicates the vibrissal papilla. **f,g,** Primary SKPs spheres that were generated by mixing very small numbers of GFP-positive adult vibrissal cells and E18 primary skin cells and culturing in the presence of FGF2 and EGF for one week. The top panel is a phase micrograph and the bottom panel a fluorescence micrograph of the same field. Note the presence of GFP-positive spheres derived from the adult vibrissal cells (arrowhead). **h**, Immunocytochemical analysis for nestin and fibronectin in a primary sphere of cells grown under SKPs conditions from dissected adult vibrissal papillae. **i,j,** Immunocytochemical analysis for nextin and/or βIII-tubulin in adult vibrissal papilla cells differentiated for 7 (i) or 10 (j) days in FGF2, 10% serum and neurotrophins.

Figure 7. NCSC-derived cells are found in the dermal papilla of pelage follicles. Overlapping expression of β-galactosidase and the dermal papilla marker, nexin, in P5 neonatal Wnt1Cre;Rosa26R dorsal skin from pigmented (a) and (b) albino littermates. **a,** Pigmented melanin granules are deposited in the developing hair shaft by NCSCderived melanoblasts resident and interspersed in the matrix. A subpopulation of dermal papilla cells stain expression mRNAs for both β-galactosidase (arrowhead) and nexin (arrowhead). Beyond the outer root sheath, occasional β-galactosidase-positive cells can also be seen in the dermal sheath (lower arrow), a structure closely associated with dermal papillae. Single β-galactosidase-positive cells are seen emerging from the upper portions of the follicle into the interfollicle epidermis and are likely melanoblasts (upper arrow). **b,** In albino animals, faint β-galactosidase-positive cells observed in the lower matrix (arrow) are likely melanoblasts, wherease strong β-galactosidase-positive cells that coexpress nexin in the adjacent serial sections are found in the dermal papilla (arrowheads), and likely represent NCSC-derived cells that are the in vivo source of SKPs. In all cases in (b), the left, middle and right panels are photomicrographs of adjacent serial sections, with the left panel being hematoxylin and eosin-stained (H & E), and the middle and right panels being hybridization with probes specific to β-galactosidase and nexin mRNAs, respectively.

Detailed Description

A key question in the stem cell field is whether cultured multipotent adult stem cells represent bona fide endogenous multipotent precursor cells. Here we address this question, focussing on SKPs, a cultured adult stem cell from the dermis that generates both neural and mesodermal progeny. We show that SKPs derive from endogenous adult dermal precursors that share attributes with embryonic neural crest stem cells. We demonstrate that these endogenous SKPs first arise in skin during embryogenesis and persist in lower numbers into adulthood, with a niche in hair follicles. Furthermore, in transgenic mice that express β-galactosidase in neural crest progeny, tagged cells were

detected within hair follicles, including within the dermal papillae. SKPs are multipotent stem cells that colonize peripheral tissues such as skin, during embryogenesis and maintain multipotency into adulthood. In addition, there are several populations of stem cells in the skin, including keratinocyte stem cells (produce keratinocytes -skin cells) and follicle bulge stem cells (clearly involved in regulating hair growth, relationship to keratinocyte stem cells controversial). There may be a form of communication of cells between the bulge and dermal papilla during the hair growth cycle.

Experimental Results

SKPs are a distinct adult stem cell population that shares characteristics with embryonic neural crest stem cells

To characterize the possible origin of SKPs, we first compared them to stem cell populations that can generate neural and/or mesodermal progeny. Since we previously demonstrated that SKPs are distinct from mesenchymal stem cells (7), we focused upon CNS neural stem cells and neural crest stem cells (NCSCs). A direct immunocytochemical comparison of SKPs and embryonic CNS neurospheres (Fig. 1a) revealed that the two populations were distinct; both expressed nestin (Fig. 1a) and vimentin, but only SKPs expressed fibronectin, while only neurospheres contained cells expressing p75NTR (Fig. 1a). We then analysed SKPs for expression of genes associated with embryonic NCSCs. RT-PCR analysis (Fig. 1b) revealed that SKPs expressed the transcription factors slug (9), snail (10), twist (11), Pax3 (12), and Sox9 (13), all markers for embryonic NCSCs (14). With the exception of Sox9, all of these genes were expressed at lower or undetectable levels in embryonic CNS neurospheres (Fig. 1b). Interestingly, SKPs also expressed the mRNAs for Dermo-1 (15) and SHOX2 (16), which are expressed in embryonic dermis and craniofacial regions, respectively (Fig. 1c). A similar pattern of gene expression was observed in SKPs generated from embryonic, neonatal or adult skin and from SKPs passaged from 1 to 15 times. Thus, SKPs express many genes characteristic of embryonic NCSCs and/or their embryonic derivatives,

although they do not express one of the characteristic markers for previously isolated NCSCs, p75NTR17.

Two neural crest-derived cell types that are abundant in skin are melanoblasts/melanocytes and Schwann cells. To ask whether SKPs dedifferentiate from either of these two cell types following passaging in culture, we examined the first, unpassaged SKPs spheres (termed primary spheres) from neonatal rodent skin. Primary SKPs spheres did not express any of three different markers for melanoblasts and/or melanocytes, c-kit, tryp1 (Fig. 1d) or DCT. Undifferentiated SKPs also did not express the Schwann cell markers myelin basic protein (MBP), P0, p75NTR (Fig. 2d) or Sox10, making it unlikely that they were generated by dedifferentiation of either of these two cell types. In addition to their expression of transcription factors seen in embryonic NCSCs, SKPs also differentiated into cell types that are exclusively neural crest-derived during embryogenesis, including peripheral catecholaminergic neurons and Schwann cells. For catecholaminergic neurons, SKPs were differentiated for one to three weeks under conditions used to differentiate embryonic NCSCs into peripheral neurons. Immunocytochemical analysis revealed that a subpopulation of cells with the morphology of neurons coexpressed the panneuronal markers βIII-tubulin and neurofilament M (NFM) (Fig. 2c), as well as proteins typical of peripheral neurons, including p75NTR (Fig. 2c), peripherin (data not shown, Fig. 2a), and the catecholaminergic markers tyrosine hydroxylase and dopamine-β-hydroxylase (Fig. 2c). This expression was confirmed by RT-PCR (Fig. 2a) and Western blot analysis (Fig. 2b). Similar results have been observed for embryonic and neonatal SKPs, and for SKPs at passages ranging from 1 to 20.

With regard to Schwann cells, we have previously reported that SKPs differentiate into bipolar cells coexpressing GFAP and CNPase, consistent with a Schwann cell phenotype (7). Further characterization of these bipolar cells demonstrated that they also expressed S100beta and p75NTR (Fig. 2e). When these cells were differentiated in the presence of forskolin to elevate intracellular cAMP, a treatment that induces expression of peripheral myelin-specific genes in bona fide Schwann cells, the

SKP-derived cells also expressed myelin basic protein (MBP) and P0 (Fig. 2d,e). Coculture of these SKPs-derived Schwann cells with myelin-deficient CNS slice cultures led to the elaboration of extensive MBP-positive processes from the differentiated SKPs. Thus, SKPs can differentiate into cell types that are exclusively NCSC-derived during embryogenesis.

SKPs arise during embryogenesis and persist into adulthood

The similarities between SKPs and embryonic NCSCs suggested that SKPs might derive from neural crest precursors that invade the dermis during embryogenesis and then persist into adulthood. To test this possibility, we asked whether skin contained an endogenous precursor cell that could differentiate into neurons, a cell type never found in skin. Skin cells from embryonic (E18), neonatal or adult mice were dissociated and immediately differentiated under conditions that promote differentiation of neurons from embryonic NCSCs38. Immunocytochemistry (Fig. 3a,b) and Western blot analysis (Fig. 3c) revealed that a subpopulation of primary skin cells differentiated directly into cells with the morphology of neurons that coexpressed βIII-tubulin, NFM and p75NTR, a phenotype very similar to that of SKPs-derived peripheral neurons. Moreover, these differentiated skin cells expressed tyrosine hydroxylase, a marker for peripheral catecholaminergic neurons that is not detectable in embryonic skin itself (Fig. 3c). The number of neurons generated in these experiments was much higher from embryonic than from adult skin.

We then asked when SKPs could first be isolated from skin. To perform these experiments, we confirmed that at low cell densities, one primary skin cell gave rise to one primary, clonal SKPs sphere (Fig. 4a,b). Three types of evidence supported this conclusion. First, at low densities (25000 to 100,000 cells/ml with E18 skin cells), increasing the primary cell number increased the number of SKPs spheres in a linear fashion (Fig. 4a). As cell density increased above 100,000 cells/ml, the number of SKPs spheres plateaued, potentially indicative of fusion of spheres at higher densities. Second, when a low number of primary GFP-positive skin cells were mixed with higher numbers

of unlabelled skin cells, the GFP-positive and negative cells were found in mutually exclusive SKPs populations (Fig. 6 f,g). Third, primary skin cells can be plated in methylcellulose and, under these immobilized conditions, will give rise to SKPs spheres (Fig. 4b). The number of spheres obtained using this approach is similar to that obtained by counting sphere numbers at low densities. Using the sphere-forming assay, we quantified the number of SKPs-like precursors in skin (Fig. 4c,d). This analysis demonstrated that SKPs spheres were never generated from mouse skin prior to E14.5, but that from E15 to E19 there was a burst of SKPs-forming cells in skin, which peaked at approximately 0.5 to 1% of cells in embryonic skin, and then decreased approximately 10-fold by P0. While the density of these spheres apparently decreased further into adulthood, the actual total number of SKPs-forming cells in back skin remained approximately constant (Fig. 4c,d). Like the passaged SKPs, these primary SKPs spheres expressed nestin (Fig. 1d, 4e), fibronectin and vimentin, but not p75NTR. Clonally-derived primary embryonic spheres were then differentiated and analyzed by double-label immunocytochemistry to determine whether they, like passaged SKPs, could differentiate into both neural and mesodermal progeny. Spheres were differentiated under neurogenic conditions and analyzed by immunocytochemistry for SMA and βIII-tubulin or SMA and GFAP. All of the clones gave rise to smooth muscle actin (SMA)-positive cells, and 75% of these same clones generated βIII-tubulin-positive, SMA-negative neurons (Fig. 4f). Under these conditions 15% of clones generated GFAP-positive glial cells (Fig. 4f). Thus, after E14, embryonic mouse skin contains a precursor cell that can generate neurons, and embryonic primary clonal spheres exhibit a potency similar to passaged SKPs.

Papillae of the hair and whisker follicles are one niche for endogenous SKPs

As primary and secondary SKP spheres express a distinctive panel of transcription factors, we reasoned that these markers might be useful in identifying a niche for endogenous SKPs *in vivo*. To test this possibility, we first confirmed using RT-PCR that mouse skin contained cells expressing slug, snail and twist from embryogenesis to

adulthood (Fig. 5a). To refine our analysis, we performed *in situ* hybridization for these same markers on skin sections from E14.5 to adulthood (P36). Our results demonstrated that all of these transcription factors were highly enriched in the follicle dermal papilla, a dermally-derived structure located at the base of the follicle (Fig. 6d) that has previously been proposed to contain precursor cells (27, 37). Dermal papillae of E18.5 hair follicles are characterized by robust alkaline phosphatase activity (21) and expression of nexin, an anagen-specific matrix modifying factor22 (Fig. 5b-d). Strikingly, low level expression of slug and snail was also clearly detected in these structures (Fig. 5e,f). Adult hair follicles cycle through periods of active growth (anagen), regression (catagen) and rest (telogen) (23). By analyzing dorsal skin at various time points in the hair follicle cycle, we found expression of the SKP markers in the dermal papilla to be very dynamic. Highest expression of these markers was detected in the dermal papilla of anagen follicles (Fig. 5g-j), although lower levels were observed occasionally in the associated dermal sheaths. Temporal expression of these markers appear to be slightly staggered; snail precedes slug expression and twist expression was only detected in later anagen follicles. Interestingly, no significant expression of these markers was detected in telogen follicles. Despite differences in neural crest contribution to trunk and cranial dermis, overlap of dermal (8) papilla and SKP markers seems to be conserved in vibrissae follicles (Fig. 5k,l). These data suggested that the dermal papillae represent one niche for SKPs. If this were true, then one would predict that SKPs should express markers specific for dermal papillae in skin, such as nexin (22), versican (24,25) and Wnt5a (26). As predicted, RT-PCR demonstrated that SKPs but not CNS neurospheres expressed all of these genes (Fig. 6d). We therefore asked whether hair follicles contained cells with SKPs-like properties. To perform these experiments, we utilized the adult whisker vibrissae which are amenable to dissection due to their larger size. Cells were dissociated from vibrissae, and then cultured on a substratum under the same conditions used for neuronal differentiation from primary skin cells. Under these conditions, clusters of cells proliferated which were nestin-positive and many of these differentiated into βIII-tubulinpositive cells with neuronal morphology (Fig. 6a,b). Similarly, when follicles were

cultured as explants under these conditions, βIII-tubulin-positive cells differentiated and migrated away from the explants (Fig. 6c), demonstrating that whisker follicles contained precursors with neurogenic potential. We then asked whether adult vibrissae contained cells that would grow as spheres under SKPs conditions. Since SKPs proliferation is density-dependent, we mixed genetically-tagged, GFP-positive adult rat vibrissal cells with unlabelled E16 skin cells, and then cultured these in suspension in the presence of FGF2 and EGF. Over the period of one week, the vibrissal cells gave rise to GFP positive spheres of a size and morphology similar to those generated by the E18 primary skin cells (Fig. 6f,g). These studies suggest that adult vibrissae are associated with a population of endogenous SKPs precursors. To ask whether these cells were resident in the vibrissal papillae, we dissected the papillae (Fig. 6e), and then cultured the dissociated papillar cells under conditions used to generate neurons from primary skin cells. As seen for total vibrissal cells, nestin-positive papillar cells proliferated and then differentiated into βIIItubulin positive cells with the morphology of neurons (Fig. 6i,j). We also asked whether dissected papillae could give rise to SKPs spheres: whisker papillae were isolated, dissociated, and then cultured under SKPs conditions supplemented with chick embryo extract. Over the period of a week, clusters of cells grew in suspension that were morphologically similar to SKPs spheres. Immunocytochemical analysis of these papillae-derived spheres revealed that they, like SKPs, coexpressed nestin and fibronectin. Thus, three lines of evidence argue that hair and whisker follicles, and in particular the follicular papillae are one endogenous niche for SKPs; (a) follicle papillae contain cells that express the same transcription factors as do SKPs, (b) SKPs express markers that are specific to follicle papillae in skin, (c) adult vibrissae and vibrissal papillae contain nestin-positive cells that can proliferate as SKPs spheres, and that can differentiate into neurons. We believe that these findings, together with previous reports that follicle papillae form at E14-E1526, the time when SKPs are first found in skin (Fig.3c,d) provide strong support for our hypothesis that hair and whisker follicle papillae represent one endogenous SKPs niche within skin.

The dermal compartment of hair follicles contains neural crest-derived cells

The above findings argue that SKPs represent a neural crest-related precursor that colonizes the dermis during embryogenesis and that persists into adulthood, and that one niche for these precursors are follicle papillae. Thus one would predict that follicle papillae, whose embryonic origin is currently unknown, would contain neural crest-derived cells. To test this prediction, we took advantage of a genetic method that has been used previously to "tag" neural crest-derived cells *in vivo*. This approach is based upon the finding that Wnt1 is expressed specifically in neural crest stem cells in the periphery, and that mice expressing a Wnt1-Cre transgene can be crossed to those expressing a floxed RosaR26R reporter allele to mark the progeny of NCSCs with β-galactosidase (36). We therefore analyzed serial sections from dorsal skin of Wnt1Cre;RosaR26R animals by *in situ* hybridization for expression of the β-galactosidase marker gene, as well as for nexin, a marker for dermal papilla, and K1535, a marker for epidermal stem cells (Fig. 7). At P5, a stage when all pelage follicles are entering the first synchronized anagen phase, we found populations of β-galactosidase-positive cells in the dermal papilla (arrowhead, Fig. 7a,b) and occasional cells in the outer dermal sheath of hair follicles (lower arrow, Fig. 7a). These β-galactosidase-positive cells did not express K15. Faintly β-galactosidase-positive cells were also found in the epithelial compartment of the hair follicle unit, including a few cells in the matrix (Fig. 7b, arrow), bulge region and exiting to the interfollicular epidermis (Fig. 7a, upper arrowhead). These β-galactosidase-positive cells are likely progeny of NCSC-derived melanoblast stem cells previously reported to inhabit these niches (18), and/or may represent other NCSC-derived progeny present in these compartments such as Merkel cells or Schwann cells. While more readily detectable in the dermis of neonatal mice, β-galactosidase-positive cells were also identified in the dermal papilla of older mice. Thus, follicle papilla cells express NCSC markers (Fig. 5), and, as shown here by expression of a Wnt1Cre;R26R transgene, at least a subpopulation of cells within papillae are of embryonic neural crest origin,

strongly supporting the idea that a neural crest-derived population of precursor cells resides within this niche in the adult.

Overall, our results show that mammalian skin contains a multipotent adult precursor cell that is similar but not identical to embryonic NCSCs, and that can differentiate into both neural and mesodermal progeny. These endogenous precursors arise in skin during mid-embryogenesis and persist into adulthood, and can be isolated, cultured and expanded as SKPs, during which time they maintain their multipotentiality. Our findings further demonstrate that one niche for these endogenous precursor cells is the follicle dermal papilla, a niche that has been proposed to contain precursor cells (27,37), and that is a major locus for regulatory dermal/epidermal interactions (28). The implications of these findings are broad-ranging. First, these findings indicate that at least one adult tissue, the dermis, contains endogenous, surprisingly multipotent precursor cells that can differentiate into cell types that are never found in their tissue of origin, in this case neurons. This ability is not the consequence of transdifferentiation, but instead reflects the potential of these cells when they migrate into the skin during embryogenesis. These cells then persist into adulthood, with their environment presumably restricting their potential *in vivo*. Whether other multipotent adult stem cells cultured from, for example, the bone marrow5 are also representative of endogenous embryonic precursor cells remains to be determined. Second, since neural crest is the likely developmental origin of these endogenous precursors, then these findings imply that similar multipotent neural crest-derived precursors may be much more widespread than just the dermis. We ourselves have found similar sphere-forming precursor cells in a placodally-derived structure, the adult olfactory epithelium, and cells similar to SKPs have recently been isolated from another neural crest-derived structure, human dental pulp (29). It will be interesting to determine whether mesenchymal stem cells found in crest-derived tissues such as the frontal skull bones are related to SKPs. Finally, with regard to skin itself, our findings identify a precursor cell niche in skin for neural crest-derived progeny such as Merkel cells, Schwann cells, mesodermal cell types, and potentially even melanoblasts. Dysregulation of such an endogenous multipotent

precursor cell could provide an explanation for skin tumors of mixed lineages and even for genetically-defined lesions such as those found in neurofibromatosis.

The above experiments were carried out using the following methods and materials.

METHODS

Cell Culture

SKPs were cultured as described in Toma *et al.* (7). Briefly, skin from mouse embryos (E15-19), or from mouse or rat neonates (P2-P6) or adults (3 weeks and older) was dissected from the animal and cut into 2-3 mm2 pieces. Tissue was then digested with 0.1% trypsin for 10-45 min at 37°C, mechanically dissociated and filtered through a 40 μm cell strainer (Falcon). Dissociated cells were pelleted and then plated in DMEM-F12, 3:1 (Invitrogen), containing 20 ng/ml EGF and 40 ng/ml FGF2 (both Collaborative Research), hereafter referred to as proliferation media. Cells were cultured in 25 cm^2 tissue culture flasks (Falcon) in a 37°C, 5% CO2 tissue culture incubator. SKPs were passaged by mechanically dissociating spheres and then splitting 1 to 3 into new culture flasks with 75% new media and 25% conditioned media from the initial flask.

Neurospheres from the E13 embryonic telencephalon32 were cultured under the same conditions. For neuronal differentiation, SKPs spheres or primary dissociated skin cells were mechanically dissociated and plated on chamber slides (Nunc) coated with poly-Dlysine/laminin in DMEM-F12 3:1 supplemented with 40 ng/ml FGF2 and 10% FBS (BioWhittaker) for 5-7 days. Cells were then cultured an additional 5-7 days in the same media without FGF2 but with the addition of 10 ng/ml NGF (Cedar Lane), 10 ng/ml BDNF (Peprotech) and 10 ng/ml NT3 (Peprotech). For Schwann cell differentiation, dissociated spheres were cultured in DMEM-F12 3:1 supplemented with 10% FBS for 7 days, then switched to the same media supplemented with 4 μM forskolin (Sigma).

For the vibrissae experiments, rat vibrissal follicles were dissected from whisker pad and excess tissue was carefully removed. The inner root sheath was opened with

tungsten needles and the papillae removed. Papillae were digested with trypsin for 15 min. at room temperature and then mechanically dissociated. Single cells were plated on 2-well chamber slides coated with poly-D-lysine/laminin/fibronectin and then cultured using the neuronal differentiation protocol described above. Alternatively, total vibrissal cells were dissociated and treated in the same way.

Sphere counts in solution were performed after seeding 25,000 – 200,000 cells/ml in uncoated 24-well tissue culture plates (Falcon) in proliferation media for 4-7 days. Methylcellulose sphere counts were performed by plating dissociated skin cells (100,000) in DMEM-F12 (3:1), 1.5% methylcellulose (Sigma), 2% B27 (Gibco-BRL), 20 ng/ml EGF and 40 ng/ml FGF2, 1 μg/ml fungizone (Invitrogen) and 1% penicillin/streptomycin. Cells were cultured in 3.5 cm plates in a 37°C, 5% CO2 incubator and sphere formation was scored after 7-10 days. Cell mixing experiments were performed by mixing dissociated E16 or E18 skin cells with GFP-tagged (31) dissociated vibrissal follicle cells 100:1 and uncoated flasks in proliferation media with 25,000 cells/ml.

Immunocytochemistry and in situ hybridization

Immunocytochemical analysis for cells was performed either using coated slides and the cytospin system (Thermo Shandon) for SKPs spheres, or on plated cells on chamber slides (Nunc) as previously described (7,32). The following primary antibodies were used: anti-nestin monoclonal, 1:400 (BD Biosciences), anti-βIII-tubulin monoclonal, 1:500 (Tuj1 clone; BABCO), anti-neurofilament-M polyclonal, 1:200 (Chemicon), anti-GFAP polyclonal, 1:200 (DAKO), anti-p75NTR polyclonal, 1:500 (Promega), anti-SMA monoclonal, 1:400 (Sigma), anti-fibronectin polyclonal, 1:400 (Sigma), anti-tryp1 polyclonal, 1:200 (Chemicon), anti-c-kit polyclonal (Cell Signaling Technology), anti-S100β monoclonal, 1:1000 (Sigma), anti-MBP polyclonal, 1:100 (Chemicon), anti-TH monoclonal, 1:200 (Chemicon). Secondary antibodies used were: Alexa488-conjugated goat anti-mouse, 1:1000 and alexa594-conjugated goat anti-rabbit, 1:1000 (both from Molecular Probes). Processing of skin samples for histological

analysis and in situ hybridization was performed as described in Mo et al. (33). Probes are as described in Mo et al., with the exception of the probe for β-galactosidase mRNA (Ambion) and that for K15 mRNA. Immunohistochemistry and alkaline phosphatase staining on skin sections was performed as previously described (34).

RT-PCR

RNA was prepared from samples using Trizol (Invitrogen), and cDNA was generated with Revertaid Reverse Transcriptase (Fermentas) as directed by manufacturer. For all cDNA synthesis a –RT control was performed. PCR reactions were carried out as follows: 92°C 2 min., 30-35 cycles of 94°C for 60 s., Gene-specific annealing temperature for 60 s. and 72°C for 60 s. PCR primers used were as follows:

Gene	Primer Sequence	Anneal Temp (°C)	Product (BP)
Pax3	ggaggcggatctagaaaggaagga cccccggaatgagatggttgaa	59	374
Slug	cgtcggcagctccactccactctc tcttcagggcacccaggctcacat	60	348
Twist	ctttccgcccacccacttcctctt gtccacgggcctgtctcgctttct	57	334
Snail	cggcgccgtcgtccttct ggcctggcactggtatctcttcac	61.5	398
Sox9	ccgcccatcacccgctcgcaatac gcccctcctcgctgatactggtg	59.5	544
P75	gtgcggggtgggctcaggact ccacaaggcccacaaccacagc	62	422
SHOX2	ccgccgccgccaagaccac tccccaaacccgctcctacaaa	63	355
DBH	acccgggggacgtactcatcac cgggaagcggacagcagaag	59	353
peripherin	gccgccaaccgcaaccat gatccggctctcctcccccttcc	61.5	333
MBP	tggccccggggacacttc gccgctgaccaccccaccat	61.1	332
P0	cctggctgccctgctcttctcttc ccccgatcactgctcccaacac	60.1	452
Dermo-1	gcggcgctacagcaagaaatc	61.4	356

	ccatgcgccacacggagaagg		
Nexin	ccacgcaaagccaagacgac	57.4	289
	gaaaccggcctgctcatcct		
Versican	tggaaggcacagcagtttacc	56.3	427
	tcatggcccacacgattcac		
Wnt-5a	ccccctcgccatgaagaagc	60.1	552
	cagccgccccacaaccagt		
GAPDH	gtcttcaccaccatggagaag	56	281
	gtgatggcatggactgtggtc		

Western blot analysis

Lysates were prepared and Western blot analysis performed as described previously (32). Equal amounts of protein (50-100 μg) were analyzed on 7.5% or 10.5% polyacrylamide gels. The primary antibodies used were anti-DβH monoclonal 1:1000 (Pharmingen), anti-peripherin polyclonal, 1:1000 (Chemicon), anti-p75NTR polyclonal, 1:1000 (Promega), anti-TH monoclonal, 1:800 (Chemicon), anti-βIII-tubulin monoclonal, 1:1000 (Tuj1 clone; BABCO), anti-NCAM monoclonal, 1:800 (Chemicon).

REFERENCES

1. Joshi, C.V. & Enver, T. Plasticity revisited. *Curr. Opin. Cell Biol.* **14**, 749-755 (2002).

2. Terada, N. et al. Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. *Nature* **416**, 542-545 (2002).

3. Wang, X. et al. Cell fusion is the principal source of bone-marrow-derived hepatocytes. *Nature* **422**, 897-901 (2003).

4. Alvarez-Dolado, M. et al. Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. *Nature* **425**, 968-973 (2003).

5. Jiang, Y. et al. Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* **418**, 41-49 (2002).

6. Clarke, D.L. et al.Generalized potential of adult neural stem cells. *Science* **288**, 1660-1663 (2000).

7. Toma, J.G. et al. Isolation of multipotent adult stem cells from the dermis of mammalian skin. *Nat. Cell Biol.* **3**, 778-784 (2001).

8. Le Douarin, N.M. The Neural Crest. Cambridge University Press, Cambridge, UK (1982).

9. Nieto, M.A., Sargent, M.G., Wilkinson, D.G. & Cooke, J. Control of cell behavior during vertebrate development by Slug, a zinc finger gene. *Science* **264**, 835-839 (1994).

10. Smith, D.E., Franco del Amo, F. & Gridley, T. Isolation of Sna, a mouse gene homologous to the Drosophila genes snail and escargot: its expression pattern suggests multiple roles during postimplantation development. *Development* **116**, 1033-1039 (1992).

11. Soo, K. et al. Twist function is required for the morphogenesis of the cephalic neural tube and the differentiation of the cranial neural crest cells in the mouse embryo. *Dev. Biol.* **247**, 251-270 (2002).

12. Conway, S.J., Henderson, D.J. & Copp, A.J. Pax3 is required for cardiac neural crest migration in the mouse: evidence from the splotch (Sp2H) mutant. *Development* **124**, 505-514 (1997).

13. Cheung, M. & Briscoe, J. Neural crest development is regulated by the transcription factor Sox9. *Development* **130**, 5681-5693 (2003).

14. Le Douarin, N.M. & Dupin, E. Cell lineage analysis in neural crest ontogeny. *J. Neurobiol.* **24**, 146-161 (1993).

15. Li, L., Cserjesi, P. & Olson, E.N. Dermo-1: a novel twist-related bHLH protein expressed in the developing dermis. *Dev. Biol.* **172**, 280-292 (1995).

16. Clement-Jones, M. et al. The short stature homeobox gene SHOX is involved in skeletal abnormalities in Turner syndrome. *Hum. Mol. Genet.* **9**, 695-702 (2000).

17. Morrison, S.J., White, P.M., Zock, C. & Anderson, D.J. Prospective identification, isolation by flow cytometry, and in vivo self-renewal of multipotent mammalian neural crest stem cells. *Cell* **96**, 737-749 (1999).

18. Nishimura, E.K. et al. Dominant role for the niche in melanocyte stem-cell fate determination. *Nature* **416**, 854-860 (2002).

19. Britsch, S. et al. The transcription factor Sox10 is a key regulator of peripheral glial development. *Genes Dev.* **15**, 66-78 (2001).

20. Monuki, E.S., Weinmaster, G., Kuhn, R. & Lemke G. SCIP: a glial POU domain gene regulated by cyclic AMP. *Neuron* **3**, 783-793 (1989).

21. Paus, R.et al. A comprehensive guide for the recognition and classification of distinct stages of hair follicle morphogenesis. *J. Invest. Dermatol.* **113**,523-32 (1999).

22. Jensen, P.J., Yang, T., Yu, D.W., Baker, M.S., Risse, B., Sun, T.T. & Lavker, R.M. Serpins in the human hair follicle. *J. Invest. Dermatol.* **114**, 917-22 (2000).

23. Fuchs, E. & Raghavan, S. Getting under the skin of epidermal morphogenesis. *Nat. Rev. Genet.* **3**, 199-209 (2002).

24. du Cros, D.L., LeBaron, R.G. & Couchman, J.R. Association of versican with dermal matrices and its potential role in hair follicle development and cycling. *J. Invest. Dermatol.* **105**, 426-31 (1995).

25. Kishimoto, J., Ehama, R., Wu, L., Jiang, S., Jiang, N. & Burgeson, R.E. Selective activation of the versican promoter by epithelial- mesenchymal interactions during hair follicle development. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 7336-41 (1999).

26. Reddy, S. et al. Characterization of *Wnt* gene expression in developing and postnatal hair follicles and identification of *Wnt5a* as a target of Sonic hedgehog in hair follicle morphogenesis. *Mech. Dev.* **107**, 69-82 (2001).

27. Lako, M., Armstrong, L., Cairns, P.M., Harris, S., Hole, N. & Jahoda, C.A. Hair follicle dermal cells repopulate the mouse haematopoietic system. *J. Cell Sci.* **115**, 3967-3974 (2002).

28. Jahoda, C.A. & Reynolds, A.J. Dermal-epidermal interactions. Adult follicle-derived cell populations and hair growth. *Dermatol. Clin.* **14**, 573-583 (1996).

29. Miura, M. et al. SHED: stem cells from human exfoliated deciduous teeth. *Proc. Natl. Acad. Sci. USA* **100**, 5807-5812 (2003).

30. Reynolds, B.A. & Weiss, S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* **255**, 1707-1710 (1992).

31. Hadjantonakis, A.K., Gertsenstein, M., Ikawa, M., Okabe, M. & Nagy, A. Generating green fluorescent mice by germline transmission of green fluorescent ES cells. *Mech. Dev.* **76**, 79-90 (1998).

32. Barnabé-Heider, F. & Miller, F.D. Endogenously produced neurotrophins regulate survival and differentiation of cortical progenitors via distinct signaling pathways. *J. Neurosci.* **23**, 5149-5160 (2003).

33. Mo, R. et al. Specific and redundant functions of Gli2 and Gli3 zinc finger genes in skeletal patterning and development. *Development* **124**, 113-123 (1997).

34. Mill, P. et al. Sonic hedgehog-dependent activation of Gli2 is essential for embryonic hair follicle development. *Genes Dev.* **17**, 282-294 (2003).

35. Liu, Y.,Lyle, S., Yang, Z. & Cotsarelis, G. Keratin 15 promoter targets putative epithelial stem cells in the hair follicle bulge. *J. Invest. Dermatol.* **121**, 963-968 (2003).

36. Jiang, X., Rowitch, D.H., Soriano, P., McMahon, A.P. & Sucov, H.M. Fate of the mammalian cardiac neural crest. *Development* **127**, 1607-1616 (2000).

37. Jahoda, C.A.B., Whitehouse, C.J., Reynolds, A.J. & Hole, N. Hair follicle dermal cells differentiate into adipogenic and osteogenic lineages. *Exp. Dermatol.* **12**, 849-859 (2003).

38. Stemple, D. L. & Anderson, D.J. Isolation of a stem cell for neurons and glia from the mammalian neural crest. *Cell* **71**, 973-985 (1992).

Claims

1. A method of producing a population of at least ten cells, wherein at least 30% of said cells are multipotent stem cells or progeny thereof and wherein said multipotent stem cells are substantially purified from a hair follicle or a dermal papilla-containing portion thereof, said method comprising the steps of:

(a) providing a hair follicle or a dermal papilla-containing portion thereof from a mammal;

(b) culturing said hair follicle or said portion in conditions under which multipotent stem cells grow and proliferate non-adherently and at least 25% of the cells that are not multipotent stem cells die or adhere to the culture substrate; and

(c) separating non-adherent cells from adherent cells and collecting non-adherent cells; and

(d) continuing culture step (b) and (c) until at least 30% of collected cells are multipotent stem cells or progeny of said multipotent stem cells.

2. The method of claim 1, wherein said step (a) further comprises dissociating said hair follicle or said portion into smaller pieces by mechanical or enzymatic disruption.

3. A method of inducing hair growth in a mammal by providing to said mammal a population of cells, wherein at least 30% of said cells are multipotent stem cells or progeny thereof and wherein said multipotent stem cells are substantially purified from a hair follicle or a dermal papilla-containing portion thereof and are capable of producing hair follicle cells.

4. A method of inducing hair growth in a mammal by providing to said mammal a population of cells, wherein at least 30% of said cells are hair follicle cells that

have differentiated from multipotent stem cells substantially purified from the dermal papillae of a hair follicle.

5. The method of any one of claims 1, 3, or 4, wherein at least 80% of the cells are multipotent stem cells substantially purified from said hair follicle or dermal papilla-containing portion thereof.

6. The method of claim 5, wherein at least 90% of the cells are multipotent stem cells substantially purified from said hair follicle or dermal papilla-containing portion thereof.

7. The method of claim 6, wherein at least 95% of the cells are multipotent stem cells substantially purified from said hair follicle or dermal papilla-containing portion thereof.

6. The method of any one of claims 1, 3, or 4, wherein said multipotent stem cells express at least one protein selected from the group consisting of nestin, WNT-1, vimentin, fibronectin, S100, slug, snail, twist, Pax3, Sox9, Dermo, and SHOX2.

7. The method of any one of claims 1, 3, or 4, wherein said multipotent stem cells do not express measurable levels of p75NTR.

8. The method of any one of claims 1, 3, or 4, wherein said multipotent stem cells do not express measurable levels of at least one protein selected from the group consisting of tyrosinase, c-kit, tryp1, DCT, MBP, P0, or SOX10.

9. The method of any one of claims 1, 3, or 4, wherein said hair follicle is from an adult mammal.

10. The method of claim 9, wherein said hair follicle is from a juvenile mammal.

11. The method of any one of claims 1, 3, or 4, wherein said mammal is a human.

12. The method of any one of claims 1, 3, or 4, wherein said multipotent stem cell is a cell that can be differentiated into a hair follicle cell under appropriate conditions.

13. The method of any one of claims 1, 3, or 4, wherein said multipotent stem cell is a cell that can be differentiated into a neuron, an astrocyte, a Schwann cell, or an oligodendrocyte under appropriate conditions.

14. The method of any one of claims 1, 3, or 4, wherein said multipotent stem cell is a cell that can be differentiated into a non-neural cell.

15. The method of claim 14, wherein said non-neural cell is a smooth muscle cell or an adipocyte.

16. The method of any one of claims 1, 3, or 4, wherein said multipotent stem cell contains a heterologous gene in an expressible genetic construct.

17. The method of claim 16, wherein said gene encodes a therapeutic protein.

18. The method of claim 17, wherein said gene encodes a protein which induces or facilitates differentiation of said stem cell.

19. The method of claim 1, wherein said population is at least one hundred cells.

20. The method of claim 3 or 4, wherein said multipotent stem cell is obtained using the method of claim 1.

21. The method of 3 or 4, wherein said multipotent stem cell is autologous.

22. The method of claim 3 or 4, wherein said donor mammal is immunologically similar to recipient mammal.

23. The method of claim 3 or 4, wherein said mammal has a condition characterized by a reduced amount of hair.

24. The method of claim 23, wherein said condition is the result of alopecia, accidental injury, damage to hair follicles, surgical trauma, burn wound, radiation therapy, chemotherapy, incisional wound, or donor site wound from skin transplant.

25. The method of claim 3 or 4, wherein said mammal has a desire to modify physical appearance.

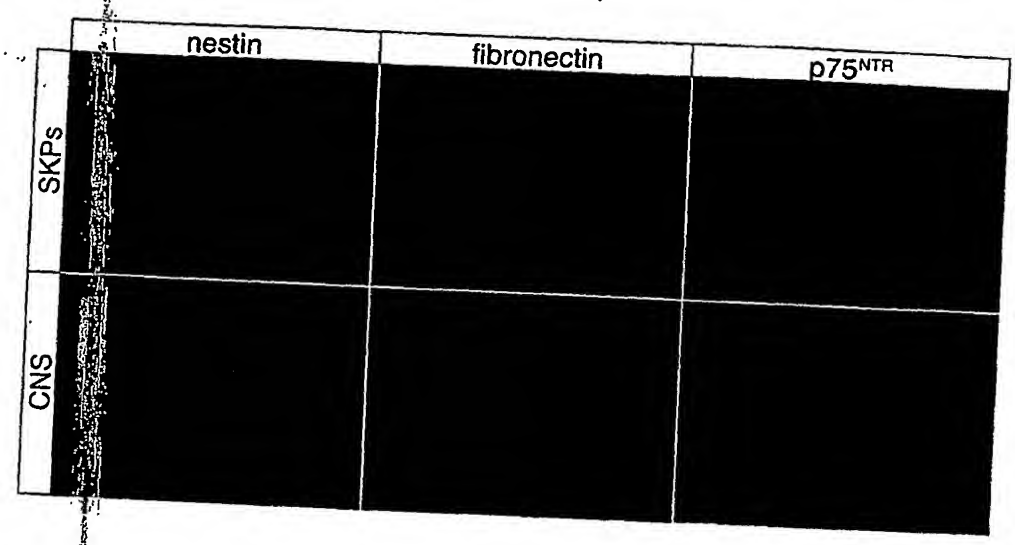
26. A kit comprising a multipotent stem cell substantially purified from a hair follicle of a postnatal mammal.

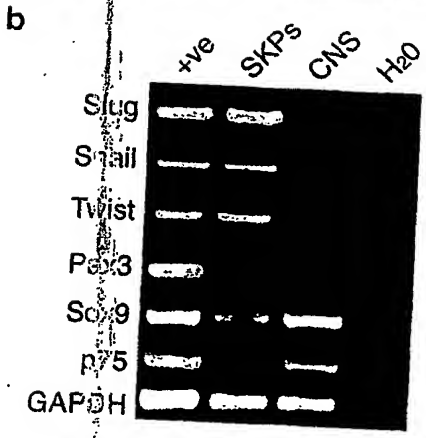
27. A kit comprising a population of cells, wherein at least 30% of said cells are multipotent stem cells substantially purified from said hair follicle.

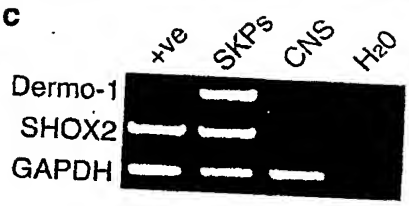
28. The kit of claim 26 or 27, further comprising instructions for inducing hair growth in a mammal.

29. A kit for the substantial purification of multipotent stem cells from the hair follicle of a postnatal mammal.

30. A kit for use in performing the method of claim 3 or 4.







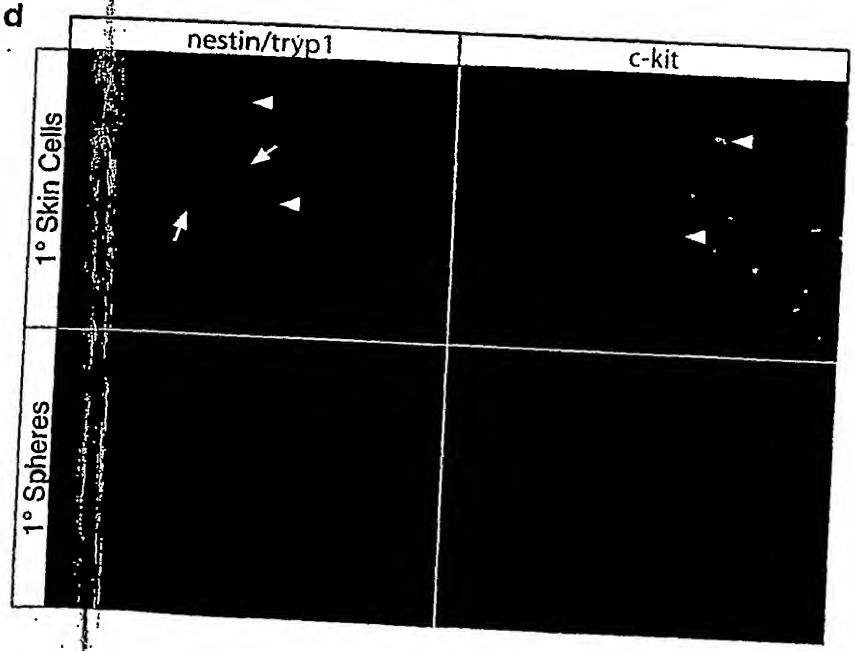


Fig 1

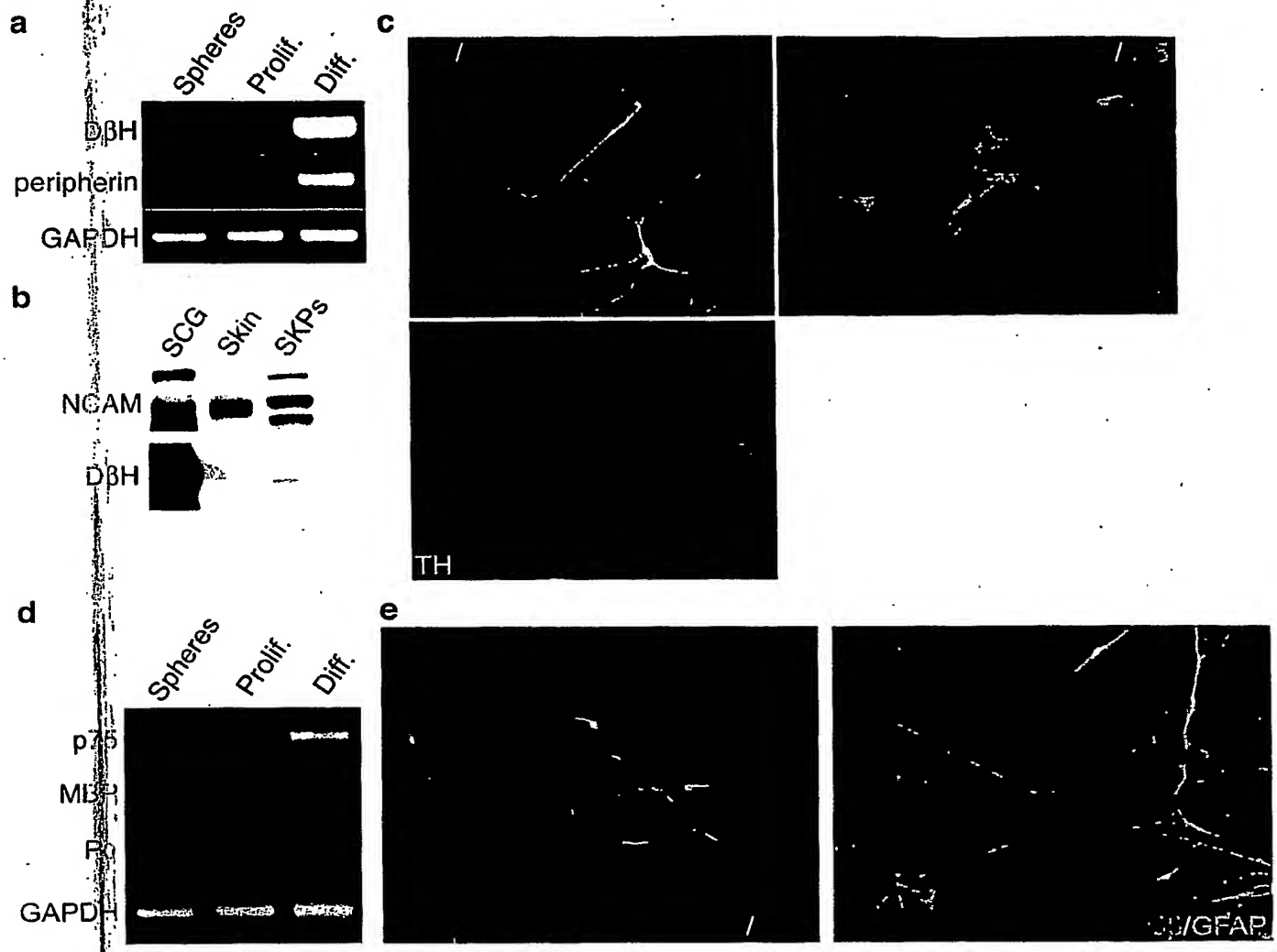


Fig 2

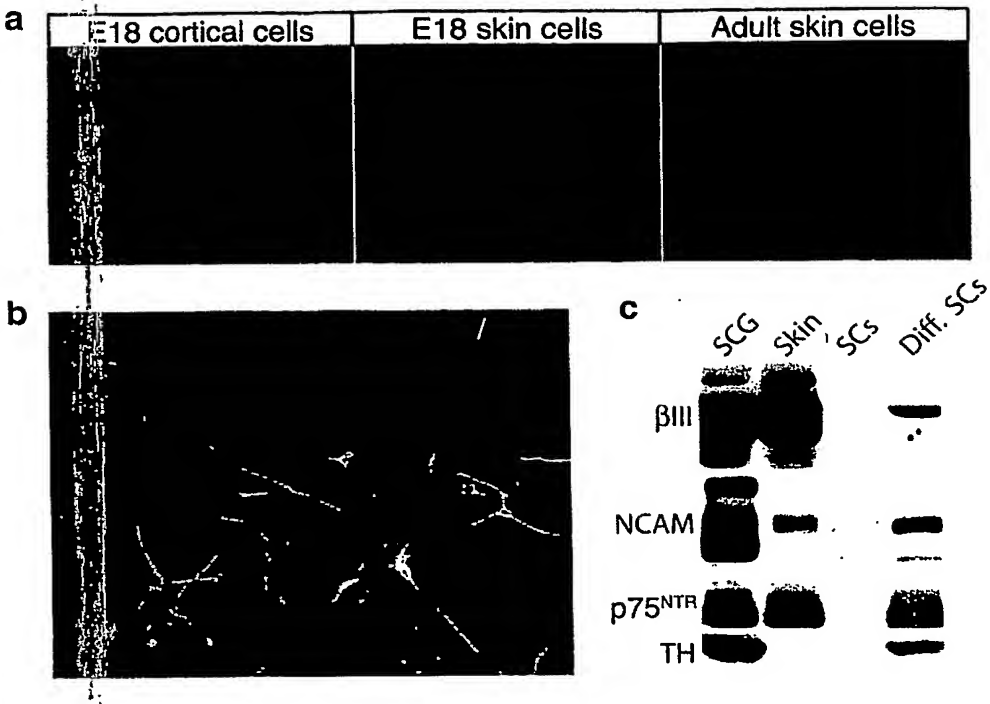
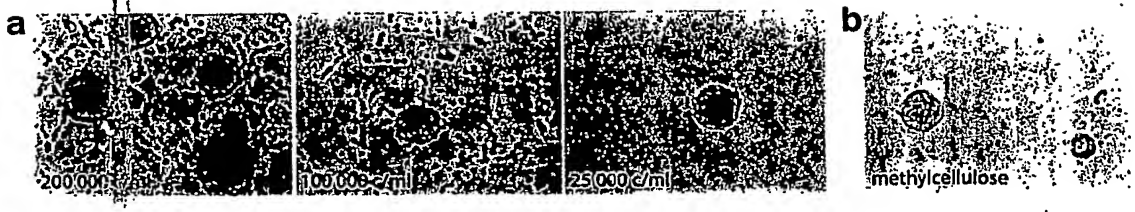
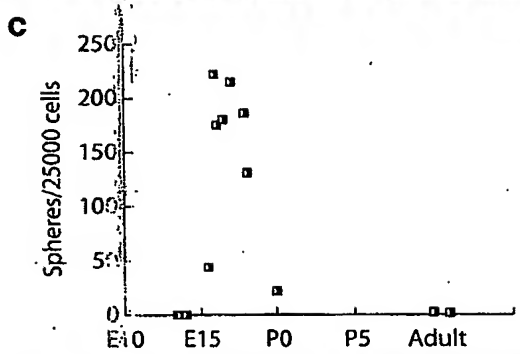
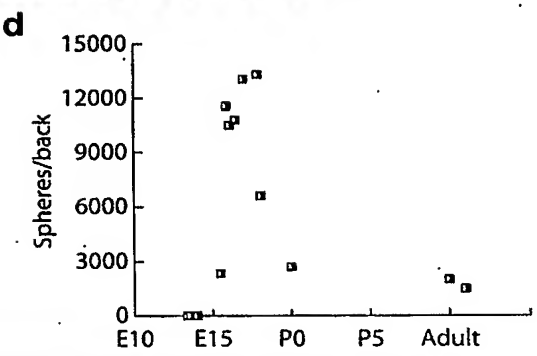


Fig 3







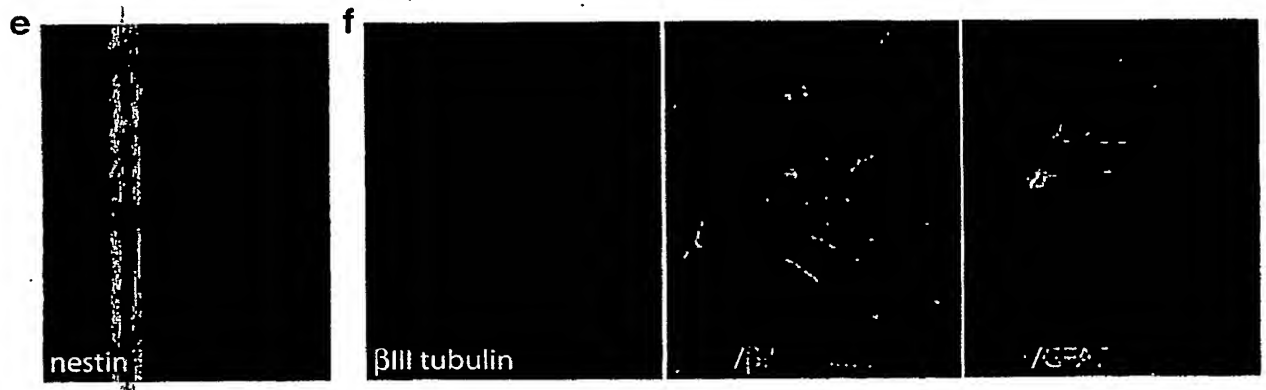


Fig 4

Fig 5

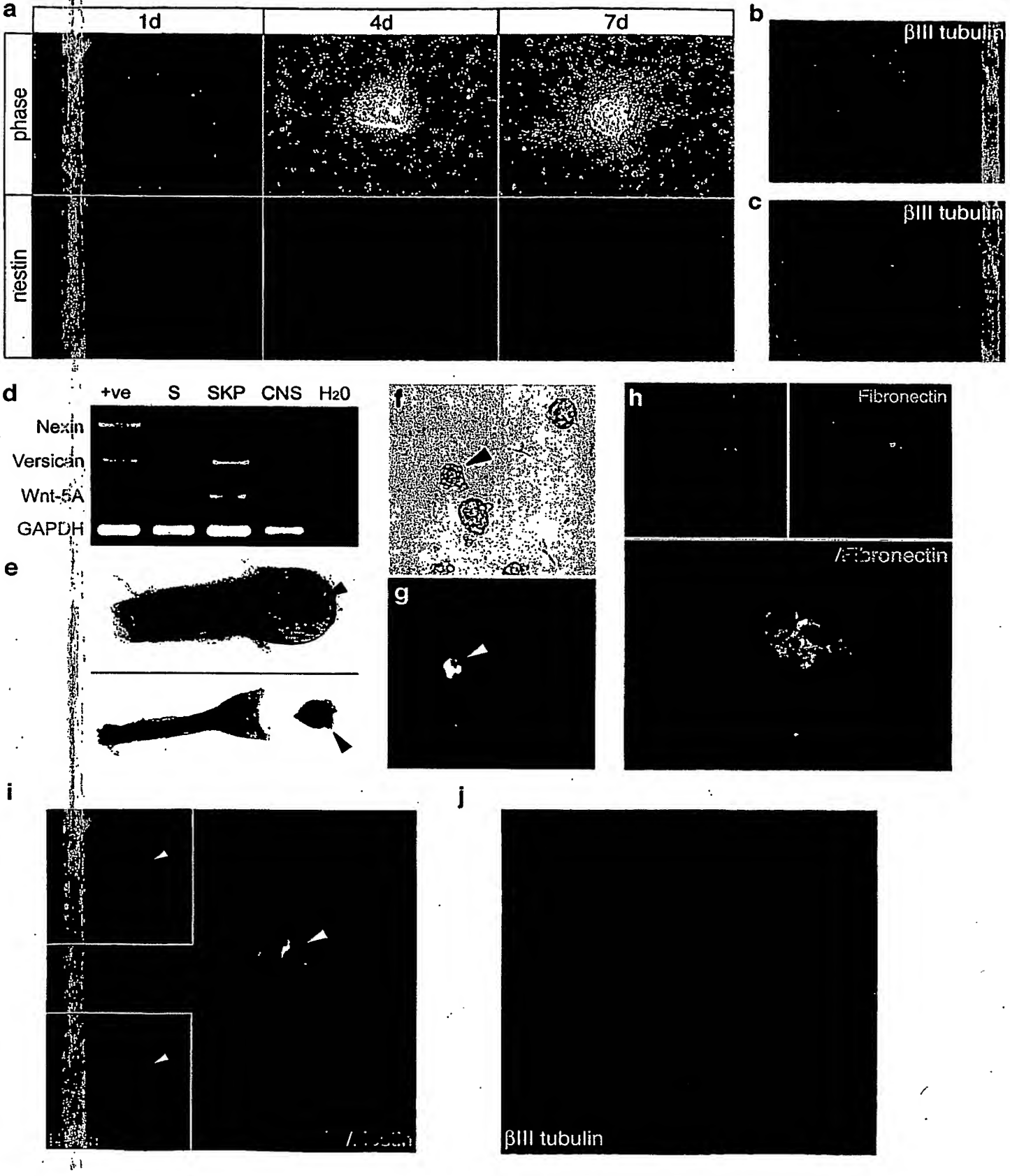
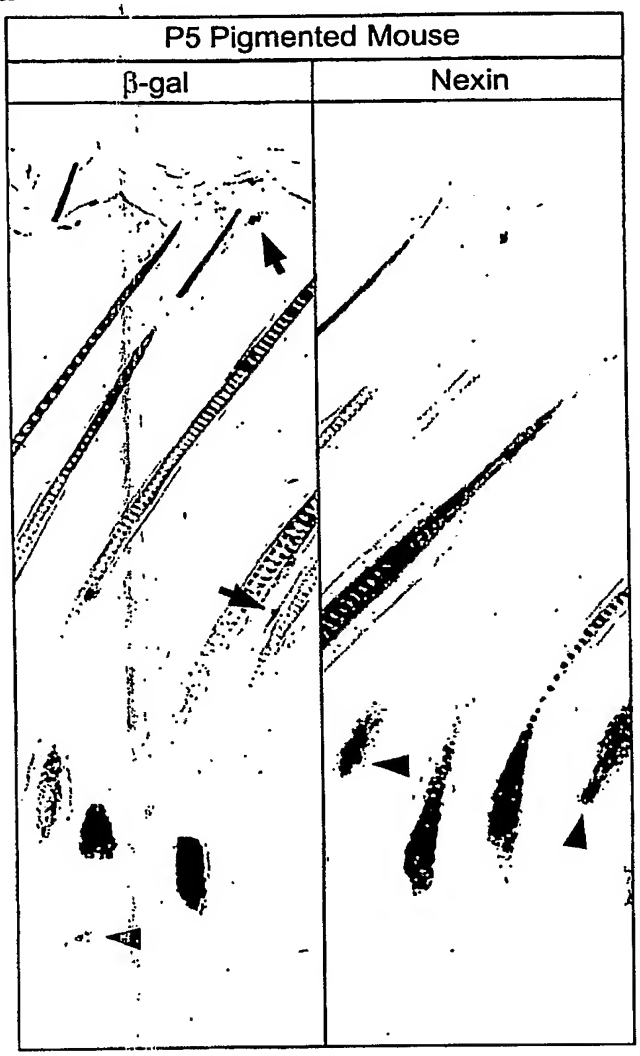


Fig 6

a



b

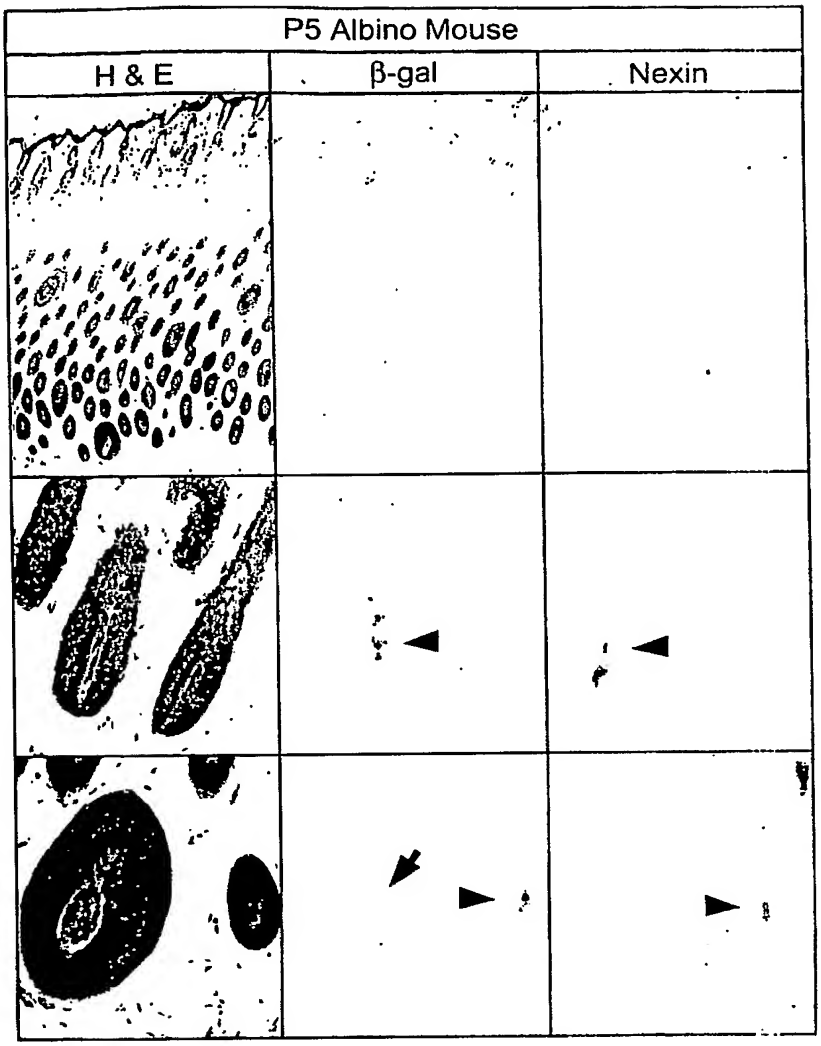


Fig 7

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.